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# Conformational Changes in Bovine-Liver Glutamate Dehydrogenase: a Spin-Label Study

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A spin-labelled analogue of *p*-chloromercuribenzoate reacts specifically with glutamate dehydrogenase. The most marked change in the properties of the spin-labelled enzyme is a fivefold decrease in the rate of reduction of the coenzyme by L-glutamate and no change in the rate of oxidation by 2-oxoglutarate. The electron spin resonance spectrum is a sensitive probe for the conformational state of the enzyme. Spin-labelled glutamate dehydrogenase in the presence of saturating concentrations of NADPH and 2-oxoglutarate or L-glutamate shows a complete conformational change while in the presence of NADP<sup>+</sup> and 2-oxoglutarate only half of the protomers have changed conformation. The conformational change upon addition of NADPH to the spin-labelled glutamate dehydrogenase in the presence of 2-oxoglutarate happens in a concerted way between 20 and 80% saturation with NADPH. One of the conformations is favoured by the activator ADP while the other is favoured by the inhibitor GTP.

Glutamate dehydrogenase catalyzes the reversible oxidation of L-glutamic acid by NAD(P)<sup>+</sup> to 2-oxoglutarate, NAD(P)H and ammonia. Important effectors of the activity are the inhibitor GTP and the activator ADP. The active enzyme has a molecular weight of about 336 000, consists of six identical protomers and is able to associate to large linear structures. Many of the kinetic and regulatory properties of bovine liver glutamate dehydrogenase are known (for review see [1]), but the function of the six subunits and the mode of action of the effectors is still poorly understood. Some of these problems may be elucidated by studies of a spin-labelled enzyme [2]. A spin-label bound to the protein may serve both as a successful reporter group [2] and as a relaxation site for obtaining distance information by NMR studies [3].

In these studies we have used a spin-labelled analogue of *p*-chloromercuribenzoate, whose formula is shown in Fig. 1. Some results of the reaction of this spin-label with glutamate dehydrogenase have been published by Jallon et al. [4]. We have studied the change of the ESR spectra upon formation of various

binary, ternary and quaternary complexes and upon titration of coenzyme. In addition NMR measurements have been performed to determine the distances between the spin-label and 2-oxoglutarate and NAD(P)<sup>+</sup>.

## MATERIALS AND METHODS

Bovine-liver glutamate dehydrogenase was obtained as a suspension in ammonium sulphate from Boehringer (Mannheim). The enzyme was dialysed at 4°C, first against 0.1 M sodium phosphate and 0.05 M EDTA at pH 8.0, then several times against 0.1 M sodium phosphate pH 7.4. After dialysing the enzyme was centrifuged at 30 000 × *g* for 20 min. Enzyme concentrations were calculated from the absorbance at 280 nm using an absorption coefficient of 0.93 cm<sup>-1</sup> ml mg<sup>-1</sup> [5]. The absorbance ratio at 280 and 260 nm was 1.96 ± 0.02. Molar concentrations

**Abbreviations.** ESR, electron spin resonance; NMR, nuclear magnetic resonance; ClHgBzOH, *p*-chloromercuribenzoic acid. ClHgBz-spin-label, spin-labelled *p*-chloromercuribenzoate (Fig. 1). In complexes ClHgBz-sl stands for spin-labelled *p*-chloromercuribenzoate and E for glutamate dehydrogenase.

**Enzyme.** Glutamate dehydrogenase or L-glutamate: NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3).

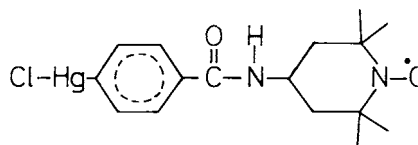


Fig. 1. Formula of the spin-labelled *p*-chloromercuribenzoate

of the enzyme are always expressed as concentration of promoters, with a molecular weight of 56000 [6]. Enzyme activity was measured spectrophotometrically by following the formation of NADH at 340 nm at 25 °C. The assay mixture was 13 mM L-glutamate and 150  $\mu$ M NAD<sup>+</sup> in 0.1 M phosphate pH 7.4. Under these conditions the specific activity was  $2.2 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ . The activity of the glutamate dehydrogenase was monitored after each titration study and was never significantly lower than at the beginning of the experiment.

Coenzymes, nucleotides and 2-oxoglutarate (monosodium salt) from Sigma and the remaining chemicals from Merck were of the highest purity available. Concentrations of nucleotides and coenzymes were estimated by ultraviolet absorbance.

All ESR and NMR studies were performed at room temperature ( $22 \pm 2^\circ\text{C}$ ). ESR spectra were recorded on a Varian E-4 spectrometer. The amount of incorporated spin-label in the enzyme was determined by running the spectrum in a flat water cell, replacing the enzyme solution by a standard solution of free spin-label (without removing the cell from the cavity) and running the spectrum of the free spin-label.

The area of both spectra were determined by double integration and the amount of spin-label incorporated was calculated by comparison with the spectrum of the free spin-label standard. Titration studies were performed in a cylindrical quartz tube with an inside diameter of 1 mm. In titration studies where the ESR spectrum changed from one type to another, the degree of change was determined by comparing the observed spectrum with spectra calculated using different ratios of the two spectral types. The NMR studies on 2-oxoglutarate were recorded using a Varian XL-100 spectrometer and on NADP<sup>+</sup> using a Bruker HX-360.

Sedimentation velocity experiments were carried out at 20 °C with 59780 rev./min in a Beckman Spinco E analytical ultracentrifuge equipped with schlieren optics. The sedimentation coefficients were determined from photographs taken at various intervals.

#### *ClHgBz-Spin-Label*

The spin-labelled *p*-chloromercuribenzoate used in this study has been used before [4], however without description of the synthesis. Our preparation of the ClHgBz-spin-label is based on the synthesis of the similar spin-label with a 5-membered nitroxide ring which has not been purified [7].

#### *Synthesis of p-Chloromercuribenzoylechloride*

3.15 g ClHgBzOH was refluxed for 4 h with 140 ml thionylchloride. The hot solution was filtered over glasswool to remove some residual ClHgBzOH. On cooling to room temperature the *p*-chloromercuriben-

zoylechloride crystallized as needles. After two days the product was obtained by vacuum filtration under nitrogen and washed with diethylether. Yield 2.10 g (63%). The carbonyl stretching of ClHgBzOH at  $1700 \text{ cm}^{-1}$  is shifted in *p*-chloromercuribenzoylechloride to  $1770 \text{ cm}^{-1}$  and a nearly equally strong absorption at  $1730 \text{ cm}^{-1}$  appeared.

#### *Synthesis of the ClHgBz-Spin-Label*

0.43 g 4-amino-2,2,6,6-tetramethylpiperidinoxy (Aldrich) was dissolved three times in freshly distilled pyridine and evaporated to dryness and then dissolved in 10 ml pyridine. This solution was added to 0.93 g *p*-chloromercuribenzoylechloride in 25 ml pyridine. The solution was stirred and became clear orange. After half an hour at room temperature it was evaporated to dryness and four times suspended in chloroform and evaporated. After suspending it again in 15 ml chloroform, the precipitate (ClHgBzOH) was centrifuged off and the orange solution was applied on a 160 g silicagel (BDH, 60–120 mesh) column ( $2.8 \times 49 \text{ cm}$ ) in chloroform. The column was washed with one half liter of chloroform and eluted with increasing concentrations of methanol (increase of 1.5% after every 250 ml). At 4.5% methanol a yellow band eluted. This band was evaporated to dryness and dissolved in hot ethanol. The spin-label precipitated and the precipitate was dissolved in hot ethanol. A small amount of white precipitate was centrifuged off. ClHgBz-spin-label crystallized from this solution as orange crystals. Yield 0.17 g (14%). Elemental analysis gave 37.2% C, 4.5% H and 5.4% N, theoretical values 37.65% C, 4.35% H and 5.49% N. The mass spectrum showed the characteristic HgCl isotope pattern at the correct mass. The carbonyl stretching is at  $1650 \text{ cm}^{-1}$ . The  $R_F$  value on thin-layer plates (aluminium sheets silicagel 60/kieselguhr F<sub>254</sub> precoated from Merck) is, with acetone, 0.75 and, with chloroform, < 0.03. The ultraviolet spectrum has a maximum at 237 nm with  $\epsilon = 20.7 \pm 0.5 \text{ mM}^{-1} \text{ cm}^{-1}$ . The ultraviolet spectrum changes upon reaction with cysteine in a manner similar to ClHgBzOH [8]; at 250 nm the  $\Delta\epsilon = 6.1 \pm 0.2 \text{ mM}^{-1} \text{ cm}^{-1}$ .

## RESULTS

#### *Reaction of ClHgBz-Spin-Label with Glutamate Dehydrogenase*

The amount of spin-label incorporated and the activity of glutamate dehydrogenase as a function of the ClHgBz-spin-label concentration is shown in Fig. 2. The course of the reaction can be divided into two parts. During the incorporation of the first mole of spin-label per mole protomer there is a sharp decrease in activity but very little, or no, visible dena-

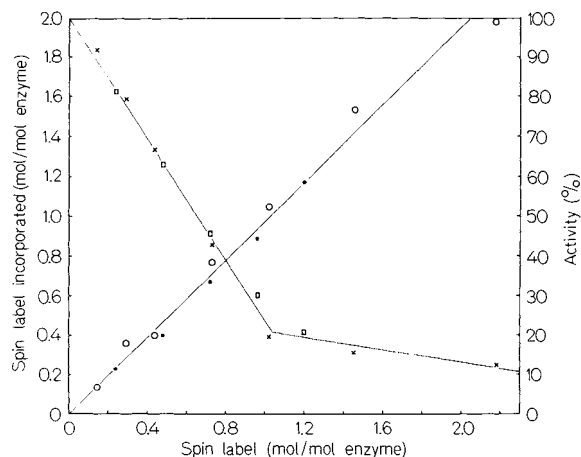


Fig. 2. The amount of ClHgBz-spin-label incorporated into enzyme protomers ( $\circ$ ,  $\bullet$ ) and the activity ( $\times$ ,  $\square$ ) as a function of ClHgBz-spin-label. The concentration of the enzyme in the experiments were 21.2 mg/ml ( $\circ$ ,  $\times$ ) and 13.0 mg/ml ( $\bullet$ ,  $\square$ ). Reactions in 0.1 M phosphate pH 7.4, at 15 °C. Activity measurements with 13 mM L-glutamate and 150  $\mu$ M NAD<sup>+</sup>

turation. Further incorporation of spin-label results in a much slower loss of activity and considerable precipitation of the enzyme. This suggests that there is a specific and relatively rapid reaction of one cysteine resulting in decreased activity followed by a slower modification of other cysteines resulting in denaturation. This is supported by the shape of the ESR spectra. Below a one-to-one labelling the ESR spectra are the same as spectrum I in Fig. 4. Above a one-to-one labelling the spectra are very similar, except for significantly broader wings of the spectra indicating nitroxides with a lower mobility. Additional information on the course of the reaction can be obtained by following the absorbance at 250 nm [8, 9]. The absorbance changes are shown in Fig. 3 for a spin-label concentration below the enzyme protomer concentration (curve I) and above the enzyme protomer concentration (curve II). Curve I follows a normal reaction curve to a steady value of absorbance at 250 nm, while curve II, after a reaction of about one to one, starts to increase strongly. When this happens there is a visible denaturation of the enzyme. If an enzyme solution is first reacted with an equimolar concentration of ClHgBzOH for 80 min, the subsequent addition of ClHgBz-spin-label results in curve III. Clearly the initial fast reaction cannot occur because the reactive cysteine has reacted already with ClHgBzOH. Similarly ClHgBzOH cannot react in the normal way if the enzyme is first reacted with ClHgBz-spin-label.

Below a one-to-one labelling there proved to be an additional contamination of a few percent quite mobile spin-label. This amount is independent of the degree of labelling. Presumably some denatured

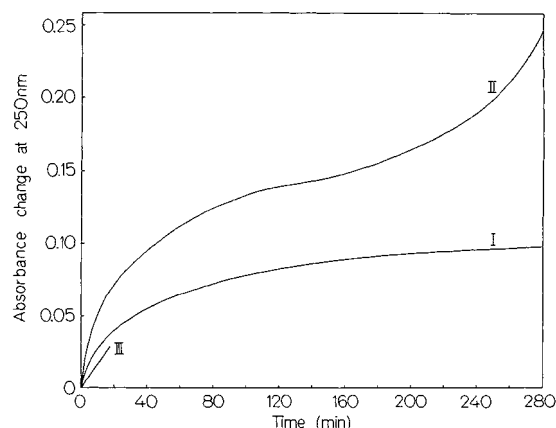


Fig. 3. Absorbance change at 250 nm during the reaction of ClHgBz-spin-label and glutamate dehydrogenase. Measurements in 0.1 M phosphate pH 7.4, at 15 °C. Enzyme concentration 1.6 mg/ml (29  $\mu$ M). ClHgBz-spin-label concentration 25  $\mu$ M (I), 45  $\mu$ M (II) and 72  $\mu$ M (III). Prior to C the enzyme was reacted during 80 min with 29  $\mu$ M ClHgBzOH

enzyme with very rapidly reacting cysteines is present. Near one-to-one labelling it hardly disturbs the spectra as can be seen in Fig. 5, but for obtaining the correct spectra (Fig. 4) it can be removed by treating the enzyme solution with active carbon.

As a result of the information presented in the above paragraph we used the following procedure for preparation of ClHgBz-spin-labelled glutamate dehydrogenase. The reaction was carried out at 15 °C (analogous to modification studies with ClHgBzOH [9]), using glutamate dehydrogenase concentrations between 13 and 22 mg/ml in 0.1 M phosphate buffer pH 7.4. ClHgBz-spin-label was added to glutamate dehydrogenase as a 20 mM solution in ethanol to a final spin-label per protomer ratio of somewhat less than one to one. The reaction time was 1.5 h followed by dialysis against 0.1 M phosphate buffer pH 7.4 overnight at 4 °C to remove the ethanol and centrifugation at 30000  $\times g$  for 20 min. Using these conditions varying levels of labelling were obtained. There was no difference in the behaviour with preparations containing various levels of label.

The spin-labelled enzyme is less stable than glutamate dehydrogenase and therefore it was prepared generally only one day before use. Since the mercaptide bond absorbs at 260 nm, the 280/260 absorbance ratio of spin-labelled glutamate dehydrogenase decreased to 1.44 for a one-to-one labelling.

#### Properties of the Spin-Labelled Enzyme

**Kinetic Properties.** The following steady-state kinetic behaviour at pH 7.4 in 0.1 M phosphate has been observed. Firstly, the activity, as measured with NAD<sup>+</sup>

and L-glutamate, decreased upon labelling. The  $K_m$  values of  $\text{NAD}^+$  increased 25–50% and the  $V$  values decreased a factor two. The same effect was found when  $\text{NADP}^+$  was used instead of  $\text{NAD}^+$ . Secondly, the activity as measured with 2-oxoglutarate,  $\text{NH}_4^+$  and  $\text{NADPH}$  or  $\text{NADH}$  (except at high concentrations, see below) was the same as found for unmodified glutamate dehydrogenase. Variable concentrations of  $\text{NAD(P)H}$  gave unchanged  $V$  and  $K_m$  values. Thirdly, the inhibition of the glutamate dehydrogenase activity by high concentrations of  $\text{NADH}$  (above 100  $\mu\text{M}$ ) was hardly present. Fourthly, the activation by  $\text{ADP}$  in the reaction of  $\text{NADPH}$ , 2-oxoglutarate and  $\text{NH}_4^+$  was abolished in the one to one spin-labelled enzyme. Fifthly, the inhibition by  $\text{GTP}$  in the reaction of  $\text{NAD(P)H}$ , 2-oxoglutarate and  $\text{NH}_4^+$  was slightly less strong: the residual activity was about a factor of two higher.

**Sedimentation Behaviour.** As a control of the association state of the spin-labelled enzyme sedimentation runs were performed. From the results, shown in Table 1, it is clear that at low enzyme concentration the association of the spin-labelled glutamate dehydrogenase is somewhat less and at high concentration it is the same as native glutamate dehydrogenase. This means that association still occurs, but the association constant is somewhat smaller. Furthermore, the normal dissociation into unimers ( $M_r$  336 000) upon addition of  $\text{GTP}$  and low concentration of  $\text{NADPH}$  also occurs in the modified enzyme.

#### The Influence of Additions on the ESR Spectrum of $\text{ClHgBz}$ -Spin-Labelled Glutamate Dehydrogenase

The addition of substrates, coenzymes and effectors change the ESR spectrum of the  $\text{ClHgBz}$ -spin-labelled glutamate dehydrogenase in the manner shown in Fig. 4. Three main types of spectra result. Spectrum type I is the spectrum without additions; it is a moderately immobile spectrum. Addition of 2-oxoglutarate and  $\text{NADPH}$  gives a change to spectrum type II, an immobile spectrum with an outer peak separation of 58 G. The presence of  $\text{GTP}$  and  $\text{NADPH}$  results in a spectrum type III, an immobile spectrum with an outer peak separation of 66 G. All other additions give spectra similar to one of these three types, sometimes with small differences, or intermediate combinations of these spectra. The results are listed in Table 2.

In addition to the results presented in Table 2 a few remarks should be made. Firstly, the type-II spectrum in the presence of  $\text{GTP}$ , belongs clearly to type II without contributions of type I or type III but it is slightly different from the type II obtained without  $\text{GTP}$ . The differences are: the wings of the spectrum are slightly broader, the left negative peak is less deep and there is a small positive peak imme-

Table 1. Sedimentation coefficients of glutamate dehydrogenase and spin-labelled glutamate dehydrogenase in 0.1 M phosphate pH 7.4 at 20 °C

For each addition the sedimentation constants of native and spin-labelled glutamate dehydrogenase were measured in the same run using a double sector cell

Enzyme concn	Additions	<i>s</i> for native enzyme	<i>s</i> for spin-labelled enzyme
mg/ml		S	
3.0	none	25.1	19.6
6.0	none	24.8	19.1
17.9	none	20.8	20.5
17.7	+ 4.7 mM GTP	20.3	20.4
17.7	+ 4.7 mM GTP + 0.29 mM NADPH	13.1	13.0
17.7	+ 4.7 mM GTP + 3.83 mM NADPH	13.1	13.2

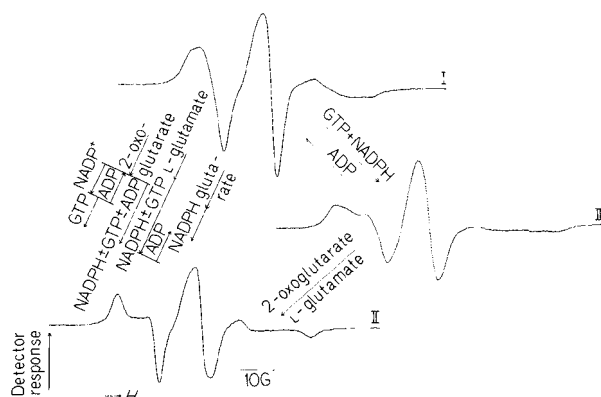


Fig. 4. ESR spectra of  $\text{ClHgBz}$ -spin-labelled glutamate dehydrogenase (0.82 spin-label per protomer). The arrows indicate the ways the three different spectra can be converted into each other. The spectra are obtained when the mentioned compounds are present at saturating concentrations. The interconversion between I and II can result in intermediate spectra as indicated by the arrows. The three spectra in this figure are recorded under the following circumstances: I, no additions; II, in the presence of 75 mM 2-oxoglutarate and 2.22 mM  $\text{NADPH}$ ; III, in the presence of 5.90 mM  $\text{GTP}$  and 2.41 mM  $\text{NADPH}$ .

diately left of the left negative peak. Secondly, complexes with both  $\text{ADP}$  and  $\text{GTP}$  are not shown in Table 2. When both effectors are present the net spectral change is in the direction of the effector present at the highest concentration in the bound complex. For example, addition of sufficient  $\text{ADP}$  to spin-labelled enzyme,  $\text{NADPH}$  and  $\text{GTP}$  changes the spectrum type III back to type I. Thirdly, additions of the non-phosphorylated coenzyme always gives the same results as mentioned in Table 2 for the phosphorylated coenzyme. The results listed in Table 2 are also represented by the arrows in Fig. 4. They suggest a complete reversibility of all changes. This reversibility can be shown by the removal of the added

Table 2. Spectra of the  $^{125}\text{I}$ HgBz-spin-labelled glutamate dehydrogenase in the presence of different compounds

The analysis of the spectra is based on the three spectral types shown in Fig. 4

Complex type	Additions	Spectral type		
		I	II	III
		%		
Binary	118 mM L-glutamate	17	83	
	114 mM 2-oxoglutarate	60	40	
	119 mM glutarate	64	36	
	1 mM NADPH	— <sup>a</sup>		
	3 mM NADP <sup>+</sup>	100		
	1.5 mM GTP	100		
	1.5 mM ADP	90	10	
Ternary <sup>b</sup>	2-oxoglutarate + NADPH		100	
	L-glutamate + NADPH		100	
	glutarate + NADPH	30	70	
	2-oxoglutarate + NADP <sup>+</sup>	30	70	
	GTP + NADPH			100
Quarter-nary <sup>b</sup>	2-oxoglutarate + NADPH + GTP		100 <sup>c</sup>	
	L-glutamate + NADPH + GTP		100 <sup>c</sup>	
	2-oxoglutarate + NADP <sup>+</sup> + GTP		100 <sup>c</sup>	
	2-oxoglutarate + NADPH + ADP		100	
	L-glutamate + NADPH + ADP	30	70	
	2-oxoglutarate + NADP <sup>+</sup> + ADP	60	40	

<sup>a</sup> Somewhat more mobile than type I

<sup>b</sup> Complexes which show the sum of the effects of the binary complexes are not shown; different concentrations are used to ensure saturating concentrations.

<sup>c</sup> The spectral type II in the presence of GTP is slightly different from type II without GTP, see text.

compounds by dialysis, which always results in a type-I spectrum.

### Titration

The changes of the ESR spectra are studied at variable concentrations of the coenzyme. The spectra resulting from a number of additions of NADPH to spin-labelled glutamate dehydrogenase and 2-oxoglutarate are shown in Fig. 5. The observed isoclinic points indicate that the spectral change occurs between only two states with no observable intermediates. The same result has been obtained for the change of spectral type I to type III.

**NADPH Titrations towards Type II.** The fractional change of the spectral type upon addition of NADPH to spin-labelled enzyme and 2-oxoglutarate is plotted in Fig. 6. The dashed line is a calculated binding curve using the dissociation constant of  $0.5 \mu\text{M}$  published by Cross [10]. The insert in Fig. 6 presents a spectrophotometric titration of NADPH to the spin-labelled glutamate dehydrogenase in the presence of 2-oxoglutarate. The curve in this insert is a binding curve calculated with a dissociation constant of  $0.5 \mu\text{M}$  and

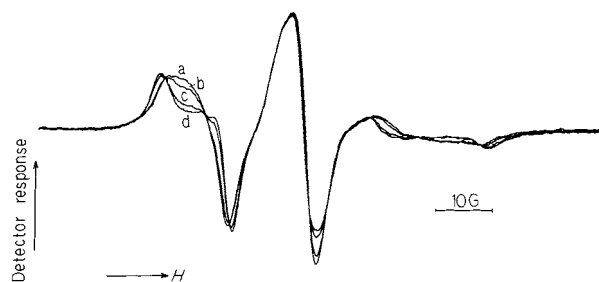


Fig. 5. ESR spectra of  $^{125}\text{I}$ HgBz-spin-labelled glutamate dehydrogenase (26.9 mg/ml, 0.70 label per protomer) in the presence of 20.9 mM 2-oxoglutarate and (a) no NADPH, (b) 0.21 mM NADPH, (c) 0.41 mM NADPH and (d) 0.83 mM NADPH

an enzyme concentration of  $95 \mu\text{M}$ . This binding curve demonstrates that a dissociation constant of  $0.5 \mu\text{M}$  is also reasonable for this modified enzyme. The change in the ESR spectral type however does not follow the binding in Fig. 5, but rather seems to reflect a conformational change dependent on the occupancy of the hexamer by NADPH. The first spectrum in the titration series depends on the concentration of 2-oxoglutarate but for all concentrations we used (20–90 mM) the change in spectral shape upon addition of NADPH was similar. Furthermore there were no indications of a dependence on the degree of labelling (0.57–0.83 label per protomer). The effect of GTP or ADP on the titration of NADPH to spin-labelled enzyme and 2-oxoglutarate is shown in Fig. 7. The titration in the presence of GTP gives the same sigmoidal curve as in the absence of GTP (see Fig. 6). Titration in the presence of ADP gives a normal binding curve with a dissociation constant of  $11 \pm 3 \mu\text{M}$  for NADPH, provided that the spectral change is directly proportional to NADPH binding. Titration of NADPH to spin-labelled glutamate dehydrogenase and 118 mM L-glutamate also gives a normal binding curve, going to spectral type II with a dissociation constant of  $5 \pm 2 \mu\text{M}$  for NADPH. The titration curve of NADPH to spin-labelled glutamate dehydrogenase and 149 mM glutarate goes from about 60% type I, 40% type II to about 30% type I, 70% type II as a normal binding curve with a dissociation constant of  $8 \pm 3 \mu\text{M}$  for NADPH. Thus, in this case, we see a half effect towards the change to type II.

**NADP<sup>+</sup> Titrations towards Type II.** A half effect is also observed upon titration of NADP<sup>+</sup> to spin-labelled glutamate dehydrogenase and 91 mM 2-oxoglutarate. The spectral change follows a normal binding curve with a dissociation constant of  $45 \pm 15 \mu\text{M}$  for NADP<sup>+</sup>. The addition of NADP<sup>+</sup> to spin-labelled glutamate dehydrogenase, 87 mM 2-oxoglutarate and 3.8 mM GTP however causes a spectral change with a normal binding curve completely to type II. The dissociation constant for NADP<sup>+</sup> is  $10 \pm 3 \mu\text{M}$ .

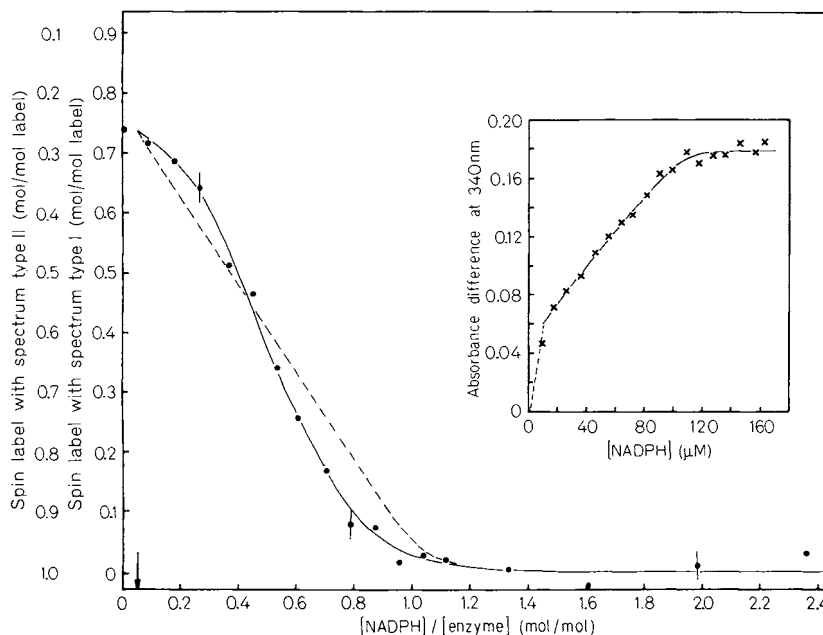


Fig. 6. Change of the ESR spectrum upon titration of NADPH to 17.6 mg/ml ClHgBz-spin-labelled glutamate dehydrogenase (0.57 label per protomer) and 48 mM 2-oxoglutarate. The curve is arbitrarily drawn through the points. The dashed line is a binding curve with a dissociation constant of 0.5  $\mu$ M, with the same begin and end point as the drawn line. The arrow denotes the amount of  $\text{NH}_4^+$  as determined spectrophotometrically by the NADPH disappearance. The curves are starting at the point where all  $\text{NH}_4^+$  has reacted. Insert: the difference in absorbance at 340 nm between NADPH added to a buffer solution and to a solution of 5.6 mg/ml spin-labelled enzyme and 24 mM 2-oxoglutarate. The first point is caused by the presence of some  $\text{NH}_4^+$ . The continuous line is a binding curve calculated with a dissociation constant of 0.5  $\mu$ M and an enzyme concentration of 95  $\mu$ M

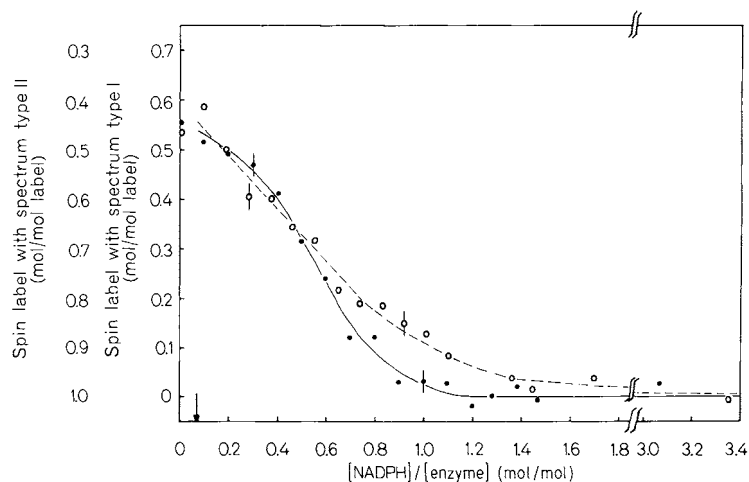


Fig. 7. Change of the ESR spectrum upon titration of NADPH to 15 mg/ml ClHgBz-spin-labelled glutamate dehydrogenase (0.69 label per protomer) in the presence of 87 mM 2-oxoglutarate and either 3.8 mM GTP (●) or 0.87 mM ADP (○). The continuous curve is an arbitrary curve representing the binding behaviour in the presence of GTP and the dashed line is a titration curve with a dissociation constant of 11  $\mu$ M fitted to the open circles. The arrow denotes the amount of  $\text{NH}_4^+$  as determined spectrophotometrically. The curves are starting at the point where all  $\text{NH}_4^+$  has reacted

**NADPH Titration towards Type III.** Only one case of conversion from type I to type III has been observed. This occurs upon addition of NADPH to spin-labelled glutamate dehydrogenase and 4.6 mM GTP. The result is shown in Fig. 8. The titration curve can be interpreted as a normal binding curve. This will be discussed in more detail.

#### Temperature Dependence

The ESR spectrum of ClHgBz-spin-labelled glutamate dehydrogenase is quite sensitive to temperature. Raising the temperature above room temperature changes the type-I spectrum into a more mobile spectrum. At low temperature it becomes like a

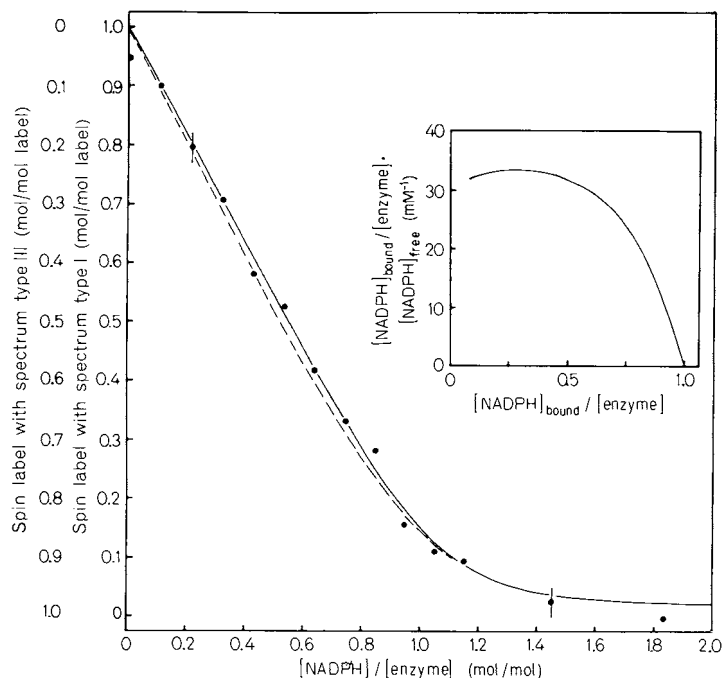


Fig. 8. Titration of NADPH to 18 mg/ml ClHgBz-spin-labelled glutamate dehydrogenase (0.65 label per protomer) in the presence of 4.6 mM GTP. The curve is the direct plot of a positive cooperative binding, which is also represented as a Scatchard plot in the insert. The dashed line is a titration curve with a dissociation constant of 7.1  $\mu$ M, the same dissociation constant as the high saturation dissociation constant in the positive cooperative binding curve

type-II spectrum, comparable to a combination of 70% type II and 30% type III. The variation in spectral type is linear with temperature between 8 °C and 37 °C. Below 4 °C essentially no further change is observed. In the presence of 96 mM 2-oxoglutarate the same spectra are obtained but at a 6 °C higher temperature. The type-II spectrum (in the presence of 2-oxoglutarate and NADPH) at low temperature is very similar to the spectrum at room temperature; the main difference is an increase of a few percent in the distance between the outer peaks. All observed spectral changes with temperature were reversible.

#### NMR Studies

NMR relaxation measurements on 2-oxoglutarate and NADP<sup>+</sup> bound to both spin-labelled enzyme and reduced (with ascorbic acid) spin-labelled enzyme have been performed to determine if the ClHgBz-spin-label reacts in the neighbourhood of the active center. The transverse relaxation time,  $T_2$ , of the bound ligands were determined as described previously [3,11]. No effect of the spin-label could be observed on either the 2-oxoglutarate protons or the adenine and nicotinamide protons of NADP<sup>+</sup>. Based on the accuracy of the measurements and a correlation time of 5 ns for the bound spin-label we calculated [3] a distance larger than 1.3 nm between the protons of 2-oxoglutarate and the nitroxide radical and larger than 1.0 nm between the adenine and nicotinamide protons of NADP<sup>+</sup> and the nitroxide radical.

#### DISCUSSION

Studies in which an enzyme is modified and alterations in the environment of the attached molecule are monitored, are reliable only if one amino acid residue per protomer is modified and the alterations observed are not caused by the modification itself. In the case of ClHgBz-spin-labelled glutamate dehydrogenase we have found that, up to one spin-label per protomer, the enzyme is stable and the ESR spectrum is uniform. Above one spin-label per protomer denaturation occurs and the ESR spectrum changes. Furthermore below one spin-label per protomer the spectral type I changes completely to type II upon addition of, for example, 2-oxoglutarate and NADPH. There is no contribution of type I in this spectrum. For a random labelling one would expect a mixture of various spectral changes on addition of ligands and indeed, at a higher degree than a one-to-one labelling, we have observed that, upon going from type I to type II, part of the ESR spectrum stays type I. In this case the alteration is also accompanied by substantial denaturation. These considerations together with the rapid decrease in activity up to one spin-label per protomer and only a slight activity decrease at a higher degree of labelling indicate that we only modify one specific residue per protomer. This is in agreement with other chemical modification studies on glutamate dehydrogenase using organic mercurials. Modification with methylmercuric hydroxide, methylmercuric iodide and methylmercuric chloride all show an alteration of



kinetic properties up to one-to-one addition and indeed, for methylmercuric iodide, it has been shown that one methylmercury per protomer is responsible for the alteration of the enzyme [12–14]. Modification studies with ClHgBzOH also show a reaction with one molecule per protomer [9], and it has been shown that cysteine-319 is modified [16]. Our results (Fig. 3) suggest that ClHgBz-spin-label reacts with the same cysteine as ClHgBzOH.

Comparison of our modification results with the studies of Jallon et al. [4] using the same spin-label shows one striking difference. Jallon et al. [4] have found a maximum labelling of 0.6 spin-label per protomer. We have found similar results when using not completely pure spin-label preparations. It appears that impurities on the order of 5% can have quite drastic effects on the labelling, probably because the impurities react with the enzyme making it less stable and causing denaturation after most of the mercuric chloride has reacted. It is possible that this denaturation results in the reduction of nitroxides by cysteines. Since Jallon et al. [4] have not given information about the quality of their label we assume that some impurities in their preparation have caused the difference between our results and theirs.

The modification of glutamate dehydrogenase with ClHgBz-spin-label has changed some steady state kinetic properties. The disappearance of the ADP activation and of the inhibition by high concentrations of NADH suggest that we have modified the common binding site [18] of ADP and NADH. The results presented in Fig. 7 suggest however that ADP still can bind and alter the spin-labelled glutamate dehydrogenase. This implies that only the kinetic effect of ADP under the conditions of our measurements, is abolished. The other marked effect is the decrease in rate of L-glutamate oxidation while the rate of the 2-oxoglutarate reduction is unchanged. Studies on the pH dependence of the ClHgBzOH-modified enzyme have shown [17] that the alteration of the activity could be explained largely by a shift in the  $pK$  of the activity-pH profile. We have not determined if a similar process could explain the altered kinetic behaviour in the spin-labelled enzyme. Whatever the reason for the changes are, the kinetic characteristics of the ClHgBz-spin-labelled glutamate dehydrogenase seem to be sufficiently similar to unmodified enzyme to use the results as characteristic of glutamate dehydrogenase.

#### *Reason for Changes in the ESR Spectra*

The changes in the mobility of the spin-label which we have observed by ESR can originate from either a direct interaction with a ligand upon binding to the enzyme or a conformational change (local or more extended) in the enzyme caused by the ligand. The fact that we see, in ternary and quaternary complexes,

effects which are not a simple addition of the binary effects indicate that we observe conformational changes. Certainly it is possible that a ligand in the ternary or quaternary complex has a different position relative to the spin-label compared to the binary complex, but this, in fact, also implies a conformational change. The NMR data also point to conformational changes as the reason for the changes in the ESR spectra inasmuch as the relaxation measurements suggest that the spin-label and the coenzyme and substrate are not close enough to interfere with one another or come in direct contact. However it should be realized that the distance between the two ends of the probe itself (i.e. the nitroxide radical and the S-Hg bond), is about 1.4 nm, while the distance between the 2-oxoglutarate protons and the nitroxide is larger than 1.3 nm and between the adenine or nicotinamide protons and the nitroxide larger than 1.0 nm. Nevertheless the spin-label is quite mobile and spends part of its time closer to the enzyme surface, possibly moving in many directions. Based on the fact that, with NMR, we measure essentially the shortest distance between the observed proton and the nitroxide (because the effect goes with  $1/r^6$ ) we expect the spin-label and substrate or coenzyme not to be in each other's way.

The conclusion that the effect of substrate and coenzyme on the ESR spectrum is a result of a conformational change is supported by adding NADPH to 2-oxoglutarate and spin-labelled glutamate dehydrogenase (Fig. 6). In the range between 0.5 and 1 NADPH bound per protomer the fraction of the protomers which have changed conformation is larger than the fraction which has bound coenzyme. This can only be interpreted as a conformational change. These results will be discussed in more detail below. In the cases where effectors are present we have clearly less proof that the spectral changes are not caused by direct interactions.

The change in spectral type reveals a series of spectra showing clear non-zero isoclinic points, as shown for the change of type I to type II in Fig. 5. As has been pointed out [15] those points are a good indication for isobestic points. This means that the spectra are a composition of spectra from only two states.

#### *ESR Spectra*

Though we have not made a detailed analysis of the ESR spectra by simulating the spectra, it is worthwhile to discuss the possible reasons for the differences in the mobility of the spin-label causing the three spectral types. The cysteine modified in this study is, in general, unreactive [1, 9]. Thus the cysteine probably has limited accessibility and we assume that after reaction the long spin-label is partly buried in the

enzyme and partly extended outside. Rotation around single bonds will allow movement of the nitroxide. From a model of the ClHgBz-spin-label it seems reasonable that the rotation axis does not coincide with the z-axis of the nitroxide (for the nitroxide coordinate system and more details of the aspects treated here, see [19]). This kind of rotation will give a reduction of the distance of the outer peaks. Furthermore it seems reasonable that the part of the spin-label outside the enzyme has a certain flexibility and, depending on the rigidity of the enzyme, even the part inside the enzyme may be quite mobile. This kind of mobility results in a movement of the spin-label in a certain cone. Neglecting for a while the internal rotation of the spin-label we can expect a kind of spectral type I when the spin-label can move in a cone with a half top angle of about  $60^\circ$  and a kind of spectral type II when the angle is reduced to about  $40^\circ$ . This change can be caused by a lower mobility of the enzyme in the area where the spin-label has reacted or by the fact that the modified cysteine has moved somewhat deeper in the enzyme upon addition of substrate and coenzyme. Clearly also the rotation around an axis making an angle with the z-axis of the nitroxide can have a large contribution to the spectra. For example when only such a rotation is present, in which the angle is about  $30^\circ$  we will obtain a kind of spectrum II. The spectral type III is even more immobile, however some more components are present.

The actual spectra depend on rotations or partial rotations of the spin-label, how fast those rotations are, how large the cone is in which the spin-label can move, how fast it moves in that cone, how large the actual hyperfine splittings of the bound spin-label are and how fast the protein itself tumbles. Too little is known to make a detailed analysis, but it is clear that going from type I to II to III the mobility decreases. This is in accordance with the temperature behaviour in which the spectral type I changes at low temperature to a spectrum composed of about 70% type II and 30% type III.

#### *Titration Studies*

As mentioned above, especially for the change of the ESR spectrum type I to type II, we are observing a conformational change. Depending on the substrates or analogues present before the addition of coenzyme the conformation is represented by an ESR spectrum in the range between purely type I and 60% type I, 40% type II. Addition of coenzyme changes the conformation, which is accompanied by an ESR spectral change to completely type II or to the half effect. These results suggest that it is, in principle, possible that either all six protomers change their conformation or only three change their conformation. The conformational change represented by the ESR

spectral change from type I to type III probably represent a complete change of all six protomers; we obtain a completely different spectral type with not more than 20% contribution of spectral type I in it. When the spectral change follows the binding curve of the coenzyme it is possible to make a best fit to the points and obtain a dissociation constant which may be compared with published data. When there is reasonable agreement it is an argument that the conformational change follows the binding of coenzyme.

The starting enzyme-ligand complexes in the titrations are in general saturated with ligand. This is clear from the binary dissociation constants: for 2-oxoglutarate, from spectrophotometric data,  $2.5 \pm 0.4$  mM [24] and, from NMR measurements  $8.5 \pm 3$  mM (unpublished results); for glutarate about the same as for 2-oxoglutarate [21]; for L-glutamate, from spectrophotometric measurements, 48 mM [28] and, from NMR measurements, 50 mM [27]; for ADP 2.7  $\mu$ M [27] and for GTP 325  $\mu$ M [27]. both from equilibrium dialysis experiments. In all of these cases when coenzyme binds to the binary complex the dissociation constant of the ligand decreases. Therefore in the ternary complexes, the enzyme is also saturated with ligand. The one exception is the experiment with L-glutamate. Here the enzyme will not be completely saturated at the beginning of the experiment. We will now discuss the different titrations.

#### *NADPH to Enzyme · Substrate*

The conformational change upon addition of NADPH to spin-labelled glutamate dehydrogenase in the presence of 2-oxoglutarate (Fig. 6) occurs almost completely between a binding of 0.2 and 0.8 NADPH per protomer. It indicates a concerted change of the conformation happening around 0.5 NADPH per protomer. The fact that first the conformational state is less altered then the actual saturation of the enzyme shows that at least this part of the curve does not fit to a simple sequential model (for a discussion of the available allosteric models see Levitzky [20] and references in it). The binding of NADPH to this complex, studied spectrophotometrically [10] in the same way as shown in Fig. 6 (insert), does not show cooperativity. This type of measurement, however, is not very accurate and it is certainly possible that the binding of NADPH to this complex is cooperative. Also it should be mentioned that even in a system which one can describe by a sequential model of conformational change, the binding does not have to be cooperative [20].

In the presence of GTP the conformational change is not altered (Fig. 7), while in the presence of ADP the conformational change still occurs but is not concerted any more. The dissociation constant of 11  $\mu$ M obtained assuming that the change in conformation

follows the binding is quite reasonable, based on the fact that ADP generally weakens the binding of the coenzyme [18,21]. These results imply that the inhibitor, GTP, does not change the subunit interactions as measured by this probe, while the activator, ADP, does alter them.

Additions of NADPH to spin-labelled enzyme in the presence of L-glutamate generates the same spectral change in a linear way. Cooperative effects in the binding as published [18,23] would not be observed, since the accuracy is too low (see also below). The dissociation constant of  $5 \pm 2 \mu\text{M}$ , assuming that the change in spectral type follows the binding, is in reasonable agreement with the published dissociation constant of  $2 \mu\text{M}$  at high NADPH concentrations [18].

The titration of NADPH to spin-labelled glutamate dehydrogenase in the presence of glutarate shows a linear change of the spectral type to only half of the effect compared to the effect in the presence of 2-oxoglutarate or L-glutamate. The change continues up to an addition of one NADPH per protomer; therefore while saturating all six sites only three protomers change conformation upon binding of NADPH. Again the studies are not accurate enough to detect any cooperativity in the binding. The NADPH dissociation constant of  $8 \pm 3 \mu\text{M}$ , obtained under the assumption that the spectral change parallels the binding, is reasonable when compared with results of the enzyme in the presence of succinate [21]. This half effect suggests that glutarate is not a good substrate analogue for glutamate dehydrogenase.

#### *NADPH to Enzyme · GTP*

The binding of NADPH to glutamate dehydrogenase in the presence of GTP is positive cooperative [18,22] and depends on the concentration of the enzyme [22]. Since the sedimentation analysis shows that the modified enzyme still dissociates upon addition of NADPH and GTP (Table 1) we expect the same positive cooperative binding for ClHgBz-spin-labelled glutamate dehydrogenase. The fact that we do not observe a sigmoidal binding curve is simply a consequence of the fact that our experimental conditions cannot distinguish between a normal binding and a cooperative binding, as is shown in Fig. 8. A positive cooperative binding using a binding curve with a Hill coefficient of 1.6 fits the data points as accurately as a non-cooperative binding curve (compare the solid and dashed curve in Fig. 8).

#### *NADP<sup>+</sup> to Enzyme · Substrate*

Addition of NADP<sup>+</sup> to ClHgBz-spin-labelled glutamate dehydrogenase in the presence of 2-oxoglutarate gives a spectral change of half the effect

compared to addition of NADPH. The results suggest a complete saturation of the enzyme with a dissociation constant of  $45 \pm 15 \mu\text{M}$ , provided that the spectral change follows the binding. The binding of one NADP<sup>+</sup> to one protomer in the presence of 2-oxoglutarate is also shown by Cross et al. [24]. The same saturation occurs in the presence of glutarate instead of 2-oxoglutarate [26]. Furthermore, one spin-labelled NAD<sup>+</sup> binds per protomer in the presence of 2-oxoglutarate [25]. Thus a complete saturation of the six protomers is again accompanied by the observed conformational change in only three protomers. The dissociation constant we have obtained is somewhat larger than the published value of  $9.6 \mu\text{M}$  [24].

The same titration in the presence of GTP yields a complete change to spectral type II and a binding curve with a dissociation constant of  $10 \pm 3 \mu\text{M}$ , suggesting a spectral change proportional with the binding of NADP<sup>+</sup>. The accuracy is too low to detect any cooperativity in the binding. Thus glutamate dehydrogenase in the presence of GTP and 2-oxoglutarate changes the conformation of all six protomers upon addition of NADP<sup>+</sup>. No change is seen upon addition of NADP<sup>+</sup> in the presence of ADP and 2-oxoglutarate, suggesting that all protomers stay in the original conformation.

#### *The Effect of ADP and GTP*

Because we do not know the functionality of the observed conformational change it is not possible to discuss the consequences of the observed half effect. It is possible however to make some conclusions about the effects of ADP and GTP. In the presence of GTP the enzyme always changes its conformation completely when the substrate or product and coenzyme are added. Thus GTP tends to bring the enzyme into the conformation represented by the ESR spectrum type II. ADP on the other hand keeps the enzyme more in the conformation obtained in the absence of coenzyme. In the case of enzyme · NADPH · 2-oxoglutarate · ADP where the enzyme has the conformation represented by spectrum type II, ADP still works against that conformation by preventing the concerted change. This antagonistic effect of ADP and GTP, each stabilising a different conformational state, is exactly what one would expect for an allosteric activator and inhibitor.

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